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Spread and population structure of *Cryphonectria parasitica* in a young chestnut orchard in Slovakia

Research Article

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Abstract: The chestnut blight pathogen *Cryphonectria parasitica* was studied in a chestnut collection composed of both seedlings and grafts derived from selected *Castanea sativa* and *C. sativa x C. crenata* trees located in south-east Slovakia, near village Príbelce on an area of approximately 3.5 ha. The study was conducted during eight years (2003-2010). During this period 133 trees were infected, which represents 59.82% of chestnut trees of all chestnut accessions. Based on the phenotype of the fungus culture and the type of cankers in the field, all isolates were determined to be virulent. No hypovirulent strains were found. No vegetative compatibility (vc) type diversity was observed. More than 130 isolates were analyzed for vc and all were in single vc type, which was identical with EU 12. All isolates assayed for mating type were MAT-1. No perithecia were observed. No significant differences were found between the proportion of cankered and dead cankered trees in seedlings and grafts of hybrid origin (*C. sativa x C. crenata*) and of *C. sativa* origin. However, particular seedlings and grafts of hybrid origin seemed to exhibit certain resistance to chestnut blight.

Keywords: *Chestnut blight • Hypovirus • Vegetative compatibility • Mating type • Castanea sativa, • C. sativa x C. crenata*

1. Introduction

Several studies have investigated the genetic diversity of invasive species of fungi, particularly plant pathogenic species. Some species of plant pathogens exhibit marked variation in diversity and population structure in different locations. In particular, some populations may be clonal, while others are sexual [1].

Analyses of *Cryphonectria parasitica* population structure and diversity are critical to the success of biological control of chestnut blight by transmission of hypovirulence [2]. Genetic diversity and population structure of the chestnut blight fungus varies considerably among populations. The diversity of vegetative-compatibility (vc) types is higher in North America than in Europe. Liu [3] found 31 vc types from a single population in Maryland, USA. High diversity also exists in China, where *C. parasitica* is a native species [4].

The northern limit of *Castanea sativa* distribution in Europe is the southern region of Slovakia. *C. parasitica* was introduced to southern Europe in the 1930s [5], but was not detected in Slovakia until 1976 [6]. The number of vc types in Slovakia is distinctly lower than in other European countries, where chestnut blight has been present for a longer period of time [7]. However, there are also areas in Europe, particularly in the south-east, where vc type diversity is very low [8].

The objectives of this study were: (i) to determine vc type diversity of *C. parasitica* in an experimental chestnut collection and to compare it with other populations of *C. parasitica*; (ii) to estimate mating type frequencies in field populations of *C. parasitica*; (iii) to detect the presence of hypovirulent strains; (iv) to determine whether perithecia of *C. parasitica* were present in the evaluated population; and (v) to assess the effect of origin of the chestnut accessions and years on disease development.

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2. Experimental Procedures

2.1 Study site and plant material

All observations were conducted in a chestnut collection located in south-east Slovakia, near the village Pribelce, at 48°12'10''N and 19°15'40''E, and elevation 285 m above sea level (centre of the collection). The chestnut collection is located on an area of approximately 3.5 ha on a slope terrain with changing inclination (11° and 8° in upper and lower sections and 16.7° in central section). The elevation difference between the lowest (267 m) and the highest point (305 m) of the collection is 38 m. The slope is exposed to the southwest.

The chestnut collection under study was established in 1998 with two-year old seedlings derived from open pollination of individual trees belonging to 13-years old half-sib and full-sib families. The majority of seedlings originated from open pollination of 10 trees belonging to full-sib families derived from three *Castanea sativa* × *Castanea crenata* crosses, in which three home mother trees *C. sativa* (sat1, sat2, sat3,) and one male tree *C. crenata* (cren.) grown in Spain, Pontevedra were used. The second group of seedlings originated from 3 open pollinated of intraspecific hybrids *Castanea sativa* × *Castanea sativa* derived from the controlled crosses whereas female parent sat2 and male parent a *C. sativa* from Spain, Pontevedra (satE) were used. The third seedling group was derived from open pollinated *C. sativa* accessions grown on the experimental plot in Arboretum Mlyňany and on chestnut populations near Pribelce [9].

One and two years after planting (in 10x10 m spacing) about one-half of seedlings were grafted. Scions for grafting were collected from the mother trees of seedlings used as stocks. The majority of grafts died during the course of several years; in 2003 the chestnut collection was composed of 162 seedlings and 68 grafts.

2.2 Sampling and isolation

Sampling was performed annually from 2003 to 2010. Bark samples (4–5 cm long) were removed by knife from the margin of chestnut blight cankers, surface sterilized in 0.15% NaClO for 20 min and subsequently washed in distilled water. Small pieces of bark tissue (*ca.* 0.5x0.5 cm) were placed on 3% malt agar and incubated at 25–27°C in the dark. Each bark sample with fruiting bodies was examined under a binocular microscope for the presence of perithecia.

2.3 Virulence assays

All *C. parasitica* isolates were assessed for the presence of hypovirus using the culture morphology assay [10]. Samples were incubated at 20–22°C under diffused daylight on the laboratory bench.

2.4 Vegetative compatibility assays

Isolates of *C. parasitica* were assayed for vegetative compatibility as described by Cortesi [11]. Isolates younger than 10 days were used for vegetative compatibility (vc) tests. The vc test was done on a potato dextrose agar green (PDAg) medium described by Powell [12]. The vegetative compatibility type was assessed after 5-7 days according to the merging/barrage response, using 31 European tester strains of *C. parasitica*.

2.5 Mating type assays

Seventy isolates were assayed for mating type using a PCR assay with primers M1-GS1 and M1-GS2-rev for type MAT-1 and primers M2-GS2 and InvA5n for type MAT-2. Culture, DNA preparation and PCR assays were conducted as previously described [13,14]. MAT-1 type M1297 and MAT-2 type M1115 strains of *C. parasitica* from the Swiss Federal Research Institute WSL were used as positive controls for the mating type assays [15].

2.6 Disease spread monitoring

Trees with newly-occurring cankers and dead blighted trees were recorded annually during the study period. Due to technical obstacles to field data acquisition, actual dates of annual monitoring varied. During the eight years (2003–2010) of the study observations were conducted on the following dates: 15.10.2003, 30.6.2004, 26.7.2005, 17.8.2006, 15.8.2007, 11.6.2008, 4.6.2009 and 28.6.2010. Survival time (time from the first occurrence of canker until death of tree) was assessed in years. If a sample tree died within the same year as disease onset, survival time was recorded as 0.5 year.

Prior to evaluation, sampled trees were grouped by their affiliation to seedlings or grafts and subsequently by their affiliation to different families. Seedlings and grafts derived from different trees of the same full-sib family were included in one group. Seedlings and grafts derived from the trees of different half-sib families of *C. sativa* origin were assigned to one group (*C. sativa* SK- mix). Five seedlings derived from a *C. crenata* tree of Spain origin were designated as a separate group within the seedling category.

Data of mean survival time of trees were evaluated by descriptive statistics and by analysis of variance using the statistical package Statgraphics Plus for Windows (StatPoint Technologies, Inc., USA). Comparison of relative frequencies of cankered and dead trees in particular categories was performed by T-test using the Excel package of MS office.

2.7 Acquisition and elaboration of climatic data

Climatic data recorded in the climate station Dolné Plachtince (3.5 km far of Príbelce) were obtained from the database of Slovak Hydrometeorological Institute in Bratislava. Daily mean, maximum and minimum air temperatures (°C) and daily rainfalls over the years 1959–2010 became a basis for calculation of both long-term (50 years) and annual (2002–2010) average values of the mentioned meteorological factors over the following period January–July (6 months).

3. Results

3.1 Observed characteristics of *Cryphonectria parasitica*

The fungus *Cryphonectria parasitica* initially occurred on the evaluated experimental plot in 2003. During the study period 133 trees were infected, which represents 59.82% of all chestnut trees on the plot. All collected samples were identified as *C. parasitica* based on symptoms and microscopic investigation of fruiting bodies.

During the culture analysis, all isolates initially had white coloured mycelia. After 2 weeks of incubation on media, the mycelia became light yellow or orange-yellow, and after 3 weeks turned red-orange. Isolates sporulated abundantly after 96-140 hours of cultivation. All isolates had the orange culture morphology described by Grente [10]. Based on phenotype of this culture and the type of cankers in the field, all isolates were considered to be virulent. No hypovirulent strains were found.

No vc type diversity was observed in the experimental plot during the study period (Table 1). More than 130 isolates of *C. parasitica* were tested for vegetative compatibility and all were single vc type, which was identical with EU 12. The presence of only one mating type was confirmed. All isolates assayed for mating type were MAT-1. Only asexual fruiting bodies pycnidia and no perithecia were observed in all examined samples.

3.2 Disease spread in relation to years

The number of trees with new cankers varied among the years (Table 1, Figure 1). The first year of observation (2003) was also first recorded occurrence of disease in the studied orchard. During the period of January-June of both 2002 and 2003, the average maximum air temperature was higher than normal (15.0°C in 2002 and 13.9°C in 2003 *versus* 13.0°C average in previous years) while average rainfall was below average (249 mm in 2002 and 150 mm in 2003 *versus* 286 mm average in previous years). This unusually dry and warm weather would result in favourable conditions for the growth and spread of *Cryphonectria parasitica*. A lower number of new infections observed in 2005, after their higher occurrence in 2004, likely correlated with the lower average mean and minimal temperature that study year (1.9°C and 7.4°C) and as well with the above-average rainfall (331 mm). On the other hand, a high occurrence of cankered accessions in 2007 correlated with high mean, maximal and minimal temperatures during the period of January–June of this year (10.9, 16.6 and 5.6°C) as well as over the period of November 2006 – February 2007 (4.0, 7.1 and 1.1°C). The warm winter weather was likely a cause of the high incidence of new cankers on accessions in February 2007. The lower number of new cankered trees in 2008 correlated with lower temperatures. The first half of 2009 was dry and relatively warm; however, the number of newly cankered trees did not increase markedly. One possible explanation for this phenomenon is that during

Year	No of cankered trees[a]	Hypovirulent isolates	isolates of EU 12 type	Mating type	
				No[b]	MAT 1
2003	6	0	6	3	3
2004	11	0	11	5	5
2005	6	0	6	4	4
2006	12	0	12	5	5
2007	24	0	24	12	12
2008	10	0	10	7	7
2009	12	0	12	5	5
2010	56	0	52	29	29
Total	137	0	133	70	70

Table 1. Hypovirulence, vegetative compatibility and mating types in the studied *Cryphonectria parasitica* population.

[a] *also number of collected isolates*
[b] *Number of isolates tested for mating type*

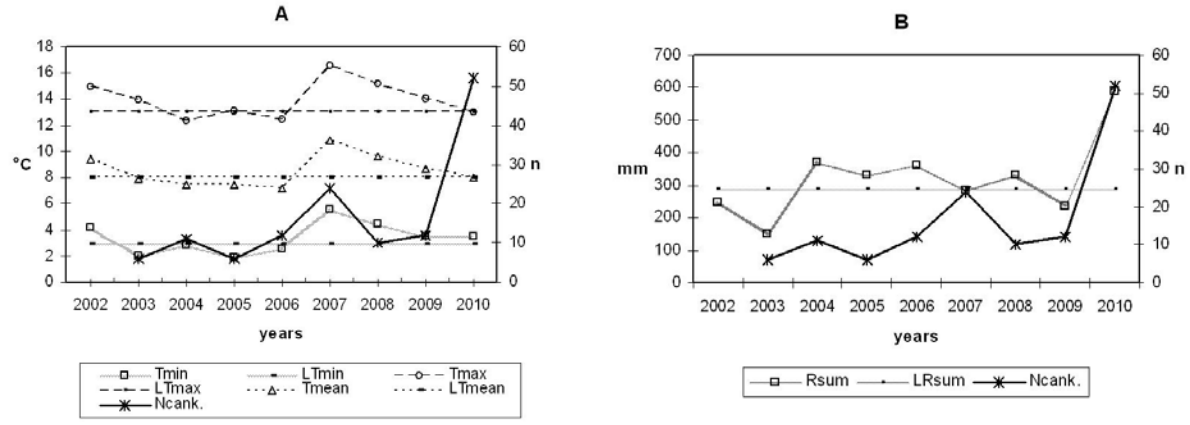

Figure 1. Frequency of cankered trees (Ncank.) related to average minimal, maximal and mean daily air temperatures (Tmin, Tmax, Tmean) (A) and sum of precipitation (Rsum) (B) for the period January–June of the years 2003–2010. Horizontal lines LTmin, LTmax, LTmean and LRsum depict 50-years-long average of the respective climatic factors.

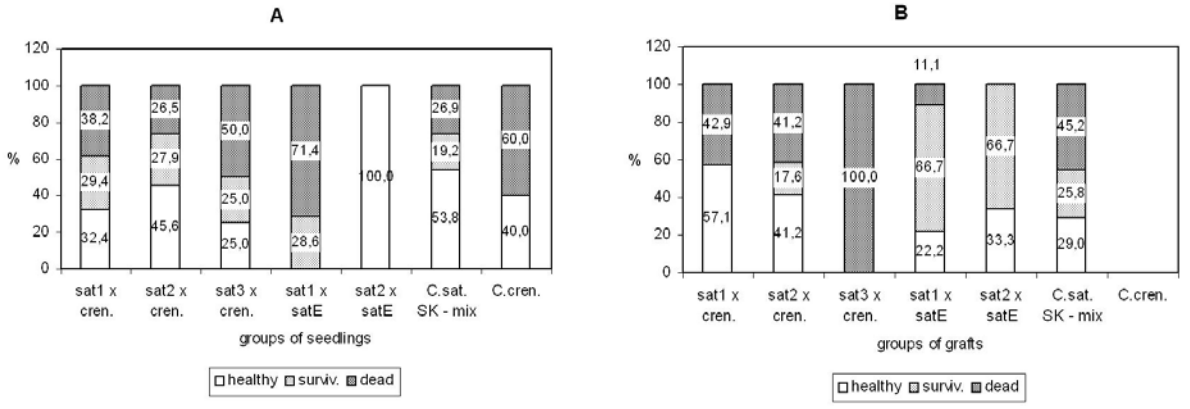

Figure 2. Relative frequency of healthy, cankered surviving and cankered dead seedlings (A) and grafts (B) calculated for period 2003–2010 in groups composed by affiliation to common half-sib family (seedlings) or full sib family or parental tree (grafts).

the hot summer months the number of cankered trees increased, but was not previously recorded. A similar situation may have occurred in 2010 when an extremely high number of newly-cankered trees was observed despite the weather conditions up until June 2008 predicting a much lower occurrence of blight (above-average rainfalls and air temperatures compared to the long-term average). In addition, the establishment and subsequent accumulation of the pathogen in the chestnut orchard is likely a cause for an increase in observed infections.

3.3 Disease incidence related to origin of the chestnut accession

The number of newly cankered and newly dead cankered trees fluctuated throughout the study regardless of accession origin. In 2010, a significant increase of cankered accessions was observed nearly in all origin categories (Table 2). No significant differences were found in percentages of both cankered and dead cankered accessions between hybrid seedlings derived from *C. sativa* x *C. crenata* and C. *sativa* seedlings (Figure 2A). Similarly, the percentage of cankered and dead hybrid grafts *C. sativa* x *C. crenata* did not significantly differ from the percentage of cankered and dead *C. sativa* grafts (Figure 2B). However, subtle differences in percentage of cankered accessions between seedlings originating from parental hybrid trees from different full-sib families suggest possible differences in their susceptibility to chestnut blight. Percentage of cankered and dead cankered seedlings derived from the trees of the family sat2 x cren was lower than in the seedlings derived from the family sat1 x cren (the families with the different female parent and the same male parent). Taking into account particular mother trees, seedlings derived from the tree sat1 x cren. showed the highest incidence of cankers as well as death. This holds also for grafts derived from the tree sat1 x cren. On the other hand, seedlings and grafts derived from the tree sat2 x cren. showed the lowest incidence

Seedlings/ grafts	Family affiliation	Cankered trees				Healthy trees	All trees
		dead		surviving			
		n	MST (yrs)	n	MST (yrs)	n	n
Seedlings	sat1 x cren.	13	1.15 abcd	10	2.40 a	11	34
	sat2 x cren.	18	1.44 bcdcf	19	2.74 a	31	68
	sat3 x cren.	2	1.00 ab	1	3.00 a	1	4
	sat1 x satE	10	0.60 a	4	1.75 a	-	14
	sat2 x satE	-		-		11	11
	C.sat. SK - mix	7	2.00 bcdef	5	2.80 a	14	26
	C.cren.	3	1.00 ab	-	-	2	5
	Total	53	1.1997 A	39	2.537 A	70	162
Grafts	sat1 x cren.	3	1.00 ab	-	-	4	7
	sat2 x cren.	7	1.29 abcde	3	1.00 a	7	17
	sat3 x cren.	1	1.00 abc	-	-	-	1
	sat1 x satE	1	3.00 cf	6	1.17 a	2	9
	sat2 x satE	-	-	2	1.50 a	1	3
	C.sat. SK - mix	14	1.29 bcde	8	1.75 a	9	31
	Total	26	1.5143 A	19	1.354 A	23	68
	Total n	79		58		93	230

Table 2. Frequency (n) and mean survival time (MST) (in years) of the trees both dead and still living after first disease onset grouped by their origin (seedling/grafts, family affiliation).

Means MST in collumns followed by the same letter do not differ statistically, sat1, sat2, sat3 – female parents C.sativa of Slovak origin, cren. – male parent C. crenata of Spain origin, satE – male parent C.sativa of Spain origin, C.sat. SK-mix – C. sativa seedlings from controlled and open pollination of different C. sativa trees

of cankers. A seedling derived from open pollination of hybrid sat2 x cren. can be considered partially resistant to chestnut blight as it was infected by chestnut blight in 2006 and in 2010 was still surviving, albeit was heavily damaged. The low number of observations of seedlings derived from F1 of sat3 x cren. does not allow accurate conclusions to be drawn regarding these accessions.

4. Discussion

The *C. parasitica* population has no diversity in the surveyed experimental plot. All *Cryphonectria parasitica* isolates collected during the eight-year study were virulent, vegetatively compatible and of MAT-1 type.

According to Benčať [16], European chestnut is grown in five cultivation subregions in Slovakia. The studied chestnut collection is situated in the Štiavnicko-krupinská subregion, where chestnut blight is present at five other localities: Horné Plachtince, Stredné Plachtince, Dolné Plachtince, Modrý Kameň, Banská Štiavnica [17, partially unpublished data]. The diversity analysis of the *C. parasitica* population at three of these localities was conducted 10 years ago [18]. The diversity of *C. parasitica* in the Štiavnicko-krupinská subpopulation was low; the H_{vc} (Shannon diversity index) ranged from 0 to 0.47. Between one and four vc types per locality were detected, with dominance of one vc type EU 12 that comprised 93% of tested isolates [18].

Usually, one to four vc types per locality/population were found in Slovakia while one vc type was detected in 7 localities in Slovakia [19,20]. The most frequent types observed were EU 12 (46.60 %) and EU 13 (33.93%). In the southwest of Slovakia, EU 13 was the dominant type (closer to the central region of chestnut blight occurrence), but in the southeast and east part of Slovakia the EU 12 type was dominant [20,21].

The results (no vc type diversity, lack of mating type polymorphism, and absence of perithecia indicating no sexual reproduction within the population) together with the fact that EU 12 is the dominat vc type in population in southern Italy and eastern Europe [7] suggest a clonal population of *C. parasitica* at the studied site. Clonality seems to be a common feature of *C. parasitica* outside the major disease areas [22]. Populations of *C. parasitica* in south-eastern Europe (Greece, Macedonia, Bulgaria,

Romania and southern Italy) are clonal with relatively low diversity [8]. In Bosnia-Herzegovina only one vc type was detected in the eastern region which was compatible with EU 12. This vc type is also dominant in south-western parts of the country [23]. All isolates from Ukraine (5 sites) were also assigned to the vc type EU 12 [24]. Only one vc type (EU 12), that is dominant throughout the country, was found in northern Greece [25]. The predominance of vc-type EU 12 and MAT-1 in *C. parasitica* populations of these countries [1] is similar to the *C. parasitica* population at the studied locality and in south-eastern and eastern Slovakia. EU 12 represents a clone of *C. parasitica*, which may be the dominant type in southern and eastern Europe either at random (*i.e.* a founder effect), or due to adaptation of this clone to the local conditions [1].

Geographically isolated founder populations of *C. parasitica* outside the natural range of chestnuts in North America have even lower genotypic diversity than populations in the centre of the host populations [26,27]. Low vc type diversity was detected in three newly established *C. parasitica* populations in northern Switzerland, outside the main range of European chestnut [22]. The number of vc types was much lower in two populations from Michigan [3], outside the natural range of American chestnut trees. A similar situation exists in the experimental plot of this study, which is located beyond the predominant range of European chestnut distribution, and may be the reason of low pathogen diversity observed. In Greece (Mt Pelion) in 1982 only a single vc type (coincident with EU 12) was reported. After 20 years the population structure in Mt Pelion did not change, the original vc type (EU 12) was the only type detected [25]. However, because *C. parasitica* was introduced to Slovakia nearly 40 years after its first occurrence in Europe, the diversity of vc types will likely increase in the future [18].

Sexual reproduction in *C. parasitica* does not occur in populations where only one mating type is present [8]. No perithecia were found in either population in Greece [25]. The lack of sexual reproduction is primarily due to the absence of mating-type MAT-2, because both types are needed for mating to occur. However, it is possible that pathogens of mating type MAT-2 could migrate from central populations, although sexual reproduction may still not occur unless environmental conditions are conducive to it [8]. Curiously, the sexual structures were found in Bulgaria and Romania; however MAT-2 was not found in any isolates from Bulgaria and was rare in Romanian populations. Sexual reproduction does not seem to have altered the population structure in either of these countries, suggesting that sexual offspring may not have contributed to the gene pool [8].

Clonal populations with little or no diversity of vegetative incompatibility, in theory, should be ideal for the rapid spread of the virus that causes hypovirulence [28]; however, no hypovirulent isolates were found on the studied plot.

The incidence of the hypovirus in southeastern Europe is variable and in most locations is low [29,30]. Variation may represent the lack of introduction of viruses to some locations where they could potentially spread rapidly in clonal populations once they are introduced [8].

The time between the introduction of *C. parasitica* and the natural appearance of hypovirus in Europe is about 20 to 30 years [7]. The disease was first observed on studied plot in 2003 [9] and in the Štiavnicko-krupinská subregion in 1991 [21]; however, no natural appearance of hypovirus has been observed to date. It can be expected that the natural occurrence of hypovirus will be detected in the future in Slovakia provided no barriers to its spreading arise.

Our results do not suggest a significant effect of hybrid origin (*C. sativa x C. crenata*) on occurrence of infection or subsequent death of trees. Resistance to chestnut blight is controlled by two to three genes in chestnut, which confounds analysis by statistical tests usually used for quantitative traits. For instance, several thousand hybrid seedlings (*C. crenata x C. sativa*) were screened for resistance to chestnut blight and individuals showing excellent production characteristics were selected [31]. Moreover, Japanese chestnut (*C. crenata*) is considered susceptible to *C. parasitica*; only high intraspecific variation within this species in some *C. crenata* genotypes can result in resistance to chestnut blight. However, resistance may be conferred by different genes than those in *C. mollissima* [32]. Although our study was not adjusted to detect resistant types, survival of trees infected with cankers suggests a certain gradient of susceptibility among *C. sativa x C. crenata* hybrids and among seedlings derived from them. Even within *C. sativa* species a continuous gradient from highly susceptible to very resistant trees to chestnut blight was reported [33].

Our results on the effect of climatic conditions on chestnut blight development are in accord with other studies, and suggest a positive effect of temperature on *C. parasitica* development. Conidial inoculations of living sprouts or excised segments between May and July resulted in the greatest incidence of infection, whereas inoculations in autumn and winter, *in vitro* as well as *in situ*, did not reveal any visible disease, demonstrating a significant seasonal effect on lesion development [34]. The results also suggest that the cankers initiated during winter and early spring, which had a low or null

expansion rate at the beginning, could increase with higher rate afterwards. This is in agreement with lesion monitoring carried out in Switzerland [35]. A direct effect of temperature on *C. parasitica* development was observed in studies on chestnut in Switzerland [36] and USA [37]. The growth rate of several strains changed relatively little between 21 and 32°C, although within this range the fastest rate generally occurred between 27 and 32°C. Growth of three virulent strains of *C. parasitica* in American chestnut trees was examined for effect of ambient temperature. There was very little difference in canker expansion rate among three experiments that covered the range of ambient summer temperatures common in Connecticut. Higher temperatures and drought could have a synergistic effect on canker development as suggested by higher rates of lesion expansion observed during summer. Severe drought conditions in 1996 within a dry inner Alpine valley (Italy) accelerated growth decline and caused death of infected *C. sativa* individuals [38].

As the absence of diversity of vegetative compatibility in *C. parasitica* population is considered a good precondition for the rapid spread of hypovirus, the biological control of chestnut blight by selected hypovirulent strains might be effective in the chestnut orchard under study.

The study on different susceptibility of chestnut accession of hybrid origin has already been extended to the controlled laboratory condition in order to select the most resistant and/or tolerant types to chestnut blight.

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